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ACQUIRED GENETIC CHANGES IN PLURIPOTENT STEM CELLS: ORIGINS AND CONSEQUENCES

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ABSTRACT (200 words)

In the twenty years since human embryonic stem cells, and subsequently induced pluripotent stem cells (collectively, pluripotent stem cells), were first described, it has become apparent that these cells may acquire genetic changes during long term culture, commonly manifest by gains or losses of particular chromosomal regions, or by mutations in certain cancer associated genes, especially *TP53*. Such changes raise concerns for the safety of products destined for clinical applications in regenerative medicine. Although acquired changes may not be present in a cell line at the start of a research program, the low sensitivity of current detection methods means that mutations may be difficult to detect if they arise but are only present in a small proportion of the cells. Nevertheless, recent work suggests that the underlying mutation rate in pluripotent stem cells is low, though they also seem to be particularly susceptible to genomic damage. This apparent contradiction can be reconciled by the observations that, in contrast to somatic cells, pluripotent stem cells are programmed to die in response to genomic damage, which may reflect the requirements of early embryogenesis. Thus, the common variants that do occur are likely rare events that offer the cells a selective growth advantage.

INTRODUCTION

Although little more than 20 years has passed since the first human embryonic stem cells (ESC) were reported (Thomson et al., 1998, Reubinoff et al., 2000), and less than 14 years since human induced pluripotent cells (iPSC) were described (Takahashi et al., 2007, Yu et al., 2007), clinical trials for regenerative medicine using derivatives of these cells are already underway or on the horizon (da Cruz et al., 2018, Schwartz et al., 2012, Song et al., 2015, Mandai et al., 2017, Barker et al., 2017). Yet over this period it has become evident that both human ESC and iPSC (collectively here denoted as PSC), although mostly diploid when first derived, may acquire genetic alterations, ranging from large scale structural modifications readily recognized as karyotypic variants through to single base pair changes on subsequent passage (**Figure 1)**. Although not the focus of this review, it is worth noting that epigenetic changes encompassing aberrations in DNA methylation, imprinting and X-chromosome inactivation have also been reported in PSC (reviewed in Bar and Benvenisty, 2019). The observation of genetic and epigenetic changes in PSC has triggered worries about the significance of such variants for the safety of PSC-based regenerative medicine (Yasuda et al., 2018, Sato et al., 2019). In particular, some recurrent genetic changes, for example mutations in *TP53*, or gains of chromosome 12p, 17q, and 20q have been associated with various cancers, notably the association of gain of chromosome 12p with embryonal carcinoma (EC) cells, the malignant counterpart of PSC (Andrews, 2002). Indeed, a planned trial of iPSC-derived retinal pigment cells to treat age related macular degeneration was halted when a point mutation was detected, although whether this particular mutation may have caused a problem was unknown (Mandai et al., 2017, Garber, 2015).

Some of the genetic variants found in PSC may well have been present in the embryos or somatic cells from which they were derived, or may have been induced during derivation (Hussein et al., 2011, Rouhani et al., 2016). The outcomes of the currently pursued in-depth studies into the effect of the choice of a starting cell type and a method of reprogramming on the overall mutational burden in iPSC (reviewed in Steichen et al., 2019) will have an important bearing on the practical applications of PSC. However, regardless of their ultimate findings, a key feature of such 'Mutations of Origin' is that they will be present in all the cells of a given PSC line and so should be readily detectable and assessed for their significance before research with a particular line is initiated. Much more problematic, and the subject of this review, are mutations that were not present initially but arise, often recurrently, during culture of the cells – 'Acquired Mutations'. Nevertheless, it is important to view these variants in perspective: many PSC lines do not acquire the commonly observed variants, or only in late passage. In a study by the International Stem Cell Initiative, 79 lines out of 122 retained a normal karyotype (Amps et al., 2011). In another study, recurrent mutants of *TP53* were detected in only five out of 140 human PSC lines (Merkle et al., 2017). On the other hand, since the sensitivity of detecting mutant cells in a mosaic culture is low (see **BOX 1**), such variant cells may lurk in cultures for a considerable time until they take over due to a selective growth advantage, or the line is subject to a population bottleneck. Further, some variants, for example gains of a small region of the long arm of chromosome 20, are particularly difficult to detect by G-banding karyotyping and may go unnoticed, even when present in a substantial proportion of the cells (Amps et al., 2011).

Over the years, many studies have sought to find ways to minimize the appearance of genetic variants in PSC. However, in such quests, it is important to recall that the appearance of the common recurrent variants depends upon two independent events, mutation followed by subsequent selection (**Figure 1**), and these can be experimentally difficult to disentangle. The mechanisms of selective growth advantage, due to the altered expression or activity of one or more genes ('Driver' genes), or to the effects of culture conditions on selection, can be relatively easily analysed by spiking wild type cultures with variant cells and monitoring their subsequent growth patterns (Olariu et al., 2010). By contrast, mutation occurs at a very low frequency so that mutations are difficult to monitor directly without expansion of the variant cells, in which case the estimates of mutation rate may be compromised by selection unless this is avoided by often cumbersome clonogenic strategies (Thompson et al., 2020).

In this review we discuss the nature of the acquired genetic variants that commonly arise during culture of human PSC cultures and consider the potential consequences of acquired genetic variants in human PSC, both for research and for clinical applications. We then discuss the mechanisms of selective growth advantage that lead to the recurrent appearance of particular variants. Finally, we focus on the underlying mechanisms of mutation in PSC; it seems that PSC differ substantially from somatic cells in both their susceptibility and response to DNA damage, which may reflect the exigencies of cell proliferation in the early embryo.

ACQUIRED GENETIC VARIATION

Karyotypic abnormalities

Traditionally, routine screening of PSC lines for the identification of genetic changes has been performed mainly by cytogenetic and molecular methods that are capable of detecting numerical and structural aneuploidies rather than DNA sequence changes (Draper et al., 2004, Amps et al., 2011). Consequently, karyotypic abnormalities are the most comprehensively catalogued genetic changes in PSC to date. In taking stock of the reports of karyotypic abnormalities in PSC over the last two decades, it is clear that the aberrant PSC karyotypes can encompass virtually any type of an abnormality, including numerical aneuploidies, such as a whole chromosome gain (trisomy) or loss (monosomy), as well as structural aneuploidies, including interstitial duplications, deletions, inversions, amplifications and translocations (Draper et al., 2004, Amps et al., 2011, Taapken et al., 2011). That said, the distribution of chromosomal aberrations appears to be non-random and certain types of variants are more commonly seen (Taapken et al., 2011, Draper et al., 2004, Amps et al., 2011, Baker et al., 2016). The first apparent bias is towards gains rather than losses of chromosomal material. Indeed, it is estimated that over 70% of all karyotypic abnormalities reported in ESC represent whole or partial chromosome gains, whereas only around 20% of reported abnormalities are losses of chromosomes or chromosomal material (Baker et al., 2016). Losses of entire chromosomes are particularly rare, representing only around 2% of reported abnormalities in PSC cultures (Baker et al., 2016). The under representation of monosomies in PSC concurs with the observation that cells in general tolerate gains of genetic material more readily than losses (Torres et al., 2008). Both unbalanced and balanced translocations, i.e. with or without the overt net gain or loss of chromosomal material, respectively, have also been reported, but unlike in certain haematological malignancies, for example, no common recurrent translocations or fusion genes have so far been associated with variant PSC (Draper et al., 2004, Amps et al., 2011, Baker et al., 2016, Assou et al., 2020). On the other hand, some chromosomes are rarely, if ever, reported as gained or lost in PSC, including chromosomes 2, 4, 19 and 21 (Amps et al., 2011, Baker et al., 2016). Finally, most striking is the observation of a consistent pattern of chromosomes affected by aneuploidy in PSC, with the majority of detected aberrations in PSC karyotypes representing gains of the whole or fragments of chromosomes 1, 12, 17, 20 and X (Baker et al., 2007, Amps et al., 2011, Nguyen et al., 2013, Baker et al., 2016, Assou et al., 2020) (**Box 2**). Of these, a particularly insidious change is the frequent gain of a small, variable region of located near the centromere of the long arm of chromosome 20 (Lefort et al., 2008, Spits et al., 2008). Often this gain is below the resolution of G-band karyotyping but, for example, it was noted by SNP array analysis in over 20% of the cell lines in the ISCI study (Amps et al., 2011, Baker et al., 2016). That the same repertoire of aneuploidies is observed across different ESC and iPSC lines and across different laboratories world-wide (Amps et al., 2011) points to the enhanced fitness of such variant cells, likely due to an

137 increased expression of one or more of the genes located on amplified chromosomes (Enver et al.,
138 2005, Ben-David et al., 2014).

139

140 **Point mutations**

141 Point mutation screening has not yet become routine in PSC maintenance and, therefore, culture
142 acquired nucleotide changes in these cells remain largely unexplored. A couple of, recent studies
143 have investigated the presence and potential recurrence of cancer-related point mutations in PSC.
144 Merkle *et al*. (Merkle et al., 2017) subjected the DNA from 140 ESC lines provided by different
145 laboratories world-wide to whole exome sequencing. After filtering out inherited polymorphisms and
146 focusing only on variants that were present in a subset of cells, as suspected culture-acquired
147 mutations, 28 of the 263 mosaic variants detected across the 140 lines were predicted to alter gene
148 function. Of these, the tumor suppressor gene *TP53* was the only gene in which mutations were
149 detected in multiple cell lines, with six different missense mutations in five independent ESC lines.
150 All of the identified *TP53* missense mutations affected cytosines of highly mutable CpG dinucleotides
151 within four of the residues encoding the DNA-binding domain of the TP53 protein, therefore rendering
152 the mutant TP53 protein inactive. Further *TP53* mutations in both ESC and iPSC were uncovered
153 by leveraging RNA-sequencing datasets from public repositories (Merkle et al., 2017, Avior et al.,
154 2019), thereby establishing *TP53* as a recurrently mutated gene in PSC. As the observed *TP53*
155 mutations represent some of the most frequent mutations in cancer (Kandoth et al., 2013) and are
156 also known to cause a familial cancer predisposition disorder, the Li-Fraumeni syndrome (Malkin et
157 al., 1990, Srivastava et al., 1990), these findings have brought into focus the need for monitoring of
158 PSC for culture-acquired *TP53* mutations.

159 Despite being the most prevalent, *TP53* mutations are not the only recurrent point mutations arising
160 in cancer-related genes upon PSC expansion (Avior et al., 2019). Recently, recurrent point
161 mutations were detected in at least 22 other genes that were previously classified within the COSMIC
162 Census database as genes with a documented cancer-related activity, including *CCND2*, *PCM1*,
163 *MYH9*, *HIF1A*, *BCL9* and *VHL* (Avior et al., 2019). Intriguingly, the mutational burden seems to differ
164 between different pluripotent states; human PSC in the naïve state, representing the pluripotent state
165 of the pre-implantation epiblast cells (Nichols and Smith, 2009), were estimated to carry four times
166 more mutations than their primed counterparts, which correspond to the pluripotent state of the post-
167 implantation epiblast (Avior et al., 2019). As the naïve samples analysed in this study were reset
168 from primed PSC, rather than derived directly from embryos, the mutational load differences may
169 not be intrinsic to different cell states, but may in fact reflect a substantial selection pressure imposed
170 on the cells during resetting to naïve pluripotency. Supporting the latter view, the genes found to
171 be mutated in the naïve cells were in pathways affected by chemical inhibitors used in resetting
172 primed cells to the naïve state(Avior et al., 2019).

173 The studies by Merkle et al (2017) and Avior et al (2019) offered an important insight into the
174 mutational landscape of PSC, albeit they focused on analysing a relatively small portion of the
175 PSC genome (i.e. the exome, which represents only about 1% of the genome). Undoubtedly,
176 uncovering the true extent and pattern of point mutations arising in PSC will require much larger,
177 ideally longitudinal datasets, and scrutiny of PSC sequence changes at a genome-wide level.
178 Important for this endeavour will be the implementation of next generation sequencing as a
179 component of routine monitoring of PSC genomes. Currently, the turnaround time and cost of
180 sequencing preclude its use as a routine screening method, but the ongoing technological
181 developments, which are driving down the cost and the data processing time, make this a feasible
182 prospect for the coming decade. Nonetheless, we must remain cognizant of the fact that the
183 reliable detection of mutations is only the first step in handling culture-acquired genetic changes.
184 A far more difficult hurdle is ascribing the functional meaning to the detected mutations, and
185 predicting their potential impact for applications of human PSC.

186

187 **Consequences for applications of human PSC**

188 The close relationship between experimentally-derived PSC and EC cells, the malignant stem cells
189 of teratocarcinomas, which occur predominantly as testicular germ cell tumors in young men
190 (Andrews, 2002, Mostofi and Price, 1973, Damjanov and Solter, 1974), and the ability of PSC to
191 produce teratomas when grown in immunodeficient mice, has always provoked concerns that cancer
192 presents a significant safety hazard for PSC based regenerative medicine. However, it is important
193 to recognize the distinction between teratomas and teratocarcinomas. Teratomas are tumors
194 containing differentiated cells without any persisting PSC. By contrast, teratocarcinomas are tumors
195 with the characteristics of teratomas that also contain undifferentiated PSC (**Figure 2**) (Damjanov
196 and Andrews, 2007b, Damjanov and Andrews, 2016). Clinically, teratocarcinomas are highly
197 malignant cancers, but they can also be effectively treated because PSC are exceptionally sensitive
198 to the chemotherapeutic agent, Cis-Platinum (Einhorn and Donohue, 1977, Oosterhuis et al., 1984,
199 Einhorn et al., 1981) as part of a standard treatment that also includes Bleomycin and Etoposide
200 (Williams et al 1987). Although some PSC do produce teratocarcinomas in which undifferentiated
201 PSC can be recognized histologically, or by outgrowths of PSC from explanted tumors (Andrews et
202 al., 2005), many of the xenograft tumors derived from PSC are better classified as teratomas (Allison
203 et al., 2018). It might be anticipated that variant PSC carrying mutations that enhance their
204 proliferative potential and, perhaps, reduce their propensity to differentiate would be more likely to
205 generate teratocarcinomas. Certainly, aneuploid PSC can produce teratocarcinomas (Andrews et
206 al., 2005). Further, the transcriptomes of ESC carrying an extra copy of chromosome 12 clustered
207 more closely with EC cells from germ cell tumors, which almost always exhibit a gain of the short
208 arm of chromosome 12, while ESC with a gain of chromosome 12 were more likely to produce
209 teratocarcinomas than the parent diploid cells from which they were derived (Ben-David et al., 2014).

210 On the other hand, in a recent ISCI study, albeit limited in scope, teratocarcinomas were produced
211 by PSC without overt karyotypic abnormalities, whereas PSC with such variants, including gains of
212 chromosome 12, produced teratomas, indicating no clear correlation between the formation of
213 teratocarcinomas and the presence of overt karyotypic changes (Allison et al., 2018). These
214 discrepancies point to the need for a more systematic study of the relationship between genotype
215 and the ability of PSC to form teratocarcinomas rather than teratomas.

216 On the other hand, regenerative medicine applications depend upon transplantation of specific
217 differentiated derivatives, not undifferentiated cells, so it is the possibility that genetic variants of PSC
218 may cause a neoplastic transformation of their derivative differentiated cells that is the greater
219 concern **(Figure 2)**. Unfortunately, there is very little direct evidence upon which to draw any definite
220 conclusions about the extent of the risks. The somatic elements in teratomas of the laboratory
221 mouse are almost always benign and non-tumorigenic (Damjanov and Solter, 1974) and this may
222 be generally true of human teratomas. However, pathologists with expertise in clinical gonadal
223 teratocarcinomas do have concerns since in the human tumors, in contrast to those of the laboratory
224 mouse, many of the differentiated elements such as neural tubes exhibit features of immaturity that
225 may be regarded as potentially neoplastic (Damjanov and Andrews, 2016). Certainly, secondary
226 somatic tumors derived from primary germ cell tumors have been found clinically, although they are
227 very rare (Mostofi and Price, 1973). Experimental, PSC-derived teratomas often also contain
228 primitive endodermal elements, which is a further concern since yolk sac carcinoma, representing
229 malignant primitive endoderm, is a well-known clinical form of germ cell tumors of the newborn
230 (Cunningham et al., 2012).

231 Although the relationship of malignant transformation of teratoma elements to particular genetic
232 variants has not been established, some of the common karyotypic variants occurring in human PSC
233 are also associated with other types of somatic cancer - for example, gains of the long arm of
234 chromosome 17 with neuroblastoma (Plantaz et al., 1997) (**BOX 2**). Further, two of the genes
235 associated with recurrent variants in human PSC, *TP53* (Merkle et al., 2017) and *BCL2L1*, the driver
236 gene of the chromosome 20q amplicon (see below) (Avery et al., 2013), which derive their selective
237 advantage for PSC from their anti-apoptotic functions, are associated with many cancers (Beroukhim
238 et al., 2010, Hainaut and Hollstein, 1999). Although the driver genes of the other common recurrent
239 variants of human PSC have yet to be identified, they provide a selective advantage because of their
240 specific effects on the undifferentiated PSC and it is entirely possible that their effects on specific
241 differentiated derivatives may be quite different. However, since many of the recurrent variants
242 involve gains or losses of large chromosomal regions, it is also possible that other 'hitch-hiker' genes
243 linked to the driver gene may also cause effects in the differentiated derivatives, separately from the
244 effects of the driver genes on the undifferentiated PSC themselves.

245 Apart from cancer, the genetic variants of PSC have the potential to cause a wide range of effects
246 on cellular physiology that could compromise the efficacy of derivative cells used in clinical

247 applications, or the production of such cells, or indeed the use of PSC in research, for example into
248 disease mechanisms. Nevertheless, there has been very little systematic consideration of these
249 issues. They were discussed by international key opinion leaders at a meeting of the International
250 Stem Cell Initiative (ISCI), at the Jackson Laboratory in 2016 (Andrews et al., 2017), and again at a
251 meeting hosted by Nature in London in 2018 (Technologies, 2018), while the Japanese regulatory
252 authorities have issued some guidelines (Research and Development Division, Health Policy
253 Bureau, The Ministry of Health, Labour and Welfare (MHLW),
254 http://www.nihs.go.jp/cbtp/sispsc/pdf/Eg.ver.Annex_0613-3_2016.pdf). However, there is no
255 international consensus about potential risk assessment and the ISCI meeting in 2016 suggested
256 the establishment of an advisory group to collate information about the common genetic variants of
257 PSC, including any evidence of their effects on cell behaviour, and linking that information to other
258 cancer and disease related genomic databases. Meanwhile, the Nature meeting strongly
259 recommended that researchers clearly document any genetic variants that may have been present
260 in cells used for particular research, so providing the data for future retrospective analysis of their
261 potential consequences. Certainly, as a minimum, the documentation should include appropriate
262 characterisation of the karyotype of the cells, and also assessment of the chromosome 20 amplicon,
263 given that these represent the most commonly observed genomic changes seen in these cells.
264

265 **SELECTION DRIVES THE APPEARANCE OF RECURRENT GENETIC VARIANTS**

266 **Growth advantage**

267 Although genetic variants may be occasionally fixed when PSC cultures are passed through a
268 population bottleneck, such as cloning, the recurrence of specific mutations within PSC populations
269 suggests that such genetic changes endow the variant cells with a selective growth advantage.
270 Consistent with this, the proportions of variant cells in a culture typically increase over time from
271 when they are first detected (Draper et al., 2004, Catalina et al., 2008, Imreh et al., 2006). Similarly,
272 in experiments designed to recapitulate the takeover of cultures by variant clones, co-mixing a small
273 proportion of commonly occurring variants with their wild-type counterparts led to a gradually
274 increased representation of variant cells in subsequent passages until they eventually dominated
275 the cultures (Olariu et al., 2010, Avery et al., 2013). Commonly, a variant may be first detected when
276 it constitutes around 5 – 10% of the cells in a culture, rising rapidly to 100% in as few as 5 passages.
277 Based on these longitudinal evaluations, the takeover of PSC cultures by variant cells has been
278 likened to Darwin's principle of natural selection, whereby the variant PSC that are best adapted to
279 particular selective conditions outcompete their neighbours and populate cultures with their own
280 progeny. The specific phenotypic features associated with genetic variants hold clues as to the
281 selective pressures operating in PSC cultures, the reduction of which is key to minimising the
282 appearance of genetic variants in expanding PSC populations.

283 In principle, genetically variant PSC could gain a selective advantage by acquiring one or several of
284 the following features: a proliferative advantage underpinned by faster cell cycle time, a decreased
285 rate of differentiation, or altered pattern of differentiation, or an increased rate of survival **(Figure 3)**.
286 Indeed, a number of studies have reported that such traits typify variant cells harbouring the
287 commonly acquired aneuploidies. For example, the growth advantage of trisomy 12 PSC was
288 attributed mainly to their significantly reduced cell cycle time, although the variant cells also displayed
289 an increased resistance to apoptosis and a reduced tendency for differentiation (Ben-David et al.,
290 2014).

291 With regard to the reduced propensity for differentiation, no studies have so far reported a total block
292 of variant ESC or iPSC to differentiation, although nullipotent EC cells are well known in the context
293 of testicular germ cell tumors (Andrews et al., 1980, Andrews et al., 1982). Rather, either a reduced
294 differentiation capacity (Fazeli et al., 2011) or a delayed differentiation dynamic (Werbowetski-
295 Ogilvie et al., 2009) compared with wild-type cells has been observed. In some instances,
296 genetically variant PSC appeared to yield alternative cell types to wild-type cells exposed to the
297 same set of differentiation conditions. For example, the same differentiation protocol applied to wild-
298 type PSC and variants with a gain of the long arm of chromosome 17 resulted in mesodiencephalic
299 dopaminergic neurons or dorsal telencephalic neurons, respectively (Lee et al., 2015). Given that
300 this gain entails amplification of a large chromosomal region and, hence, increased expression of
301 most of the genes in that region (Enver et al., 2005), it is easy to envision that such extensively
302 altered gene and protein expression profiles could include changes that skew the differentiation
303 trajectory of cells. In this case, the skewed differentiation was attributed to an increased expression
304 of *WNT3* and *WNT9B* genes localized in the amplified part of chromosome 17 (Lee et al., 2015). In
305 another case, it has been reported that BCL-XL over expression perturbs SMAD and TGFβ signalling
306 in PSC with the chromosome 20q11.21 gain, resulting in impaired neurectoderm differentiation
307 (Markouli et al., 2019). Although the altered propensity for differentiation of variant cells may be a
308 mere consequence of hitch-hiker genes rather than the driver of their growth advantage, the
309 converse may also hold true if the differentiation process itself exerts selection on the differentiating
310 cells. For example, in one study, cardiac differentiation favoured cells with a gain of the long arm of
311 chromosome 20 (Laurent et al., 2011) whereas in another report Merkle et al (Merkle et al., 2017)
312 noted an enrichment of mutant *TP53* cells upon PSC differentiation. In both cases, a variant PSC
313 population was already present in the starting cultures prior to differentiation, but it is also possible
314 that variant cells may arise and be selected during the differentiation process itself.

315 Although faster cell cycle and altered differentiation have been associated with some of the recurrent
316 variants, resistance to apoptosis seems to be a frequent feature of variants commonly detected in
317 PSC cultures. This is, perhaps, not surprising given that marked sensitivity to apoptosis represents
318 one of the notable features of early-passage diploid PSC. Excessive cell death is particularly
319 prominent when PSC are grown at a low cell density (Ohgushi et al., 2010), a condition under which

320 single PSC are confronted with a series of bottlenecks preventing their clonal growth (Barbaric et al.,
321 2014). At the molecular level, propensity for apoptosis has been explained by a low apoptotic
322 threshold of PSC, governed by low expression levels of anti-apoptotic proteins and high expression
323 levels of pro-apoptotic proteins (Liu et al., 2013). In addition to preferential expression of pro-
324 apoptotic factors, PSC store a constitutively active pro-apoptotic factor BAX in the Golgi (Dumitru et
325 al., 2012). This effectively primes PSC for a rapid apoptotic response to appropriate cues. Apart
326 from hampering the efficient scale up of PSC, the severe reduction in cell numbers during culture
327 clearly creates conditions for selection of genetically variant cells capable of blunting the apoptotic
328 pathways (Avery et al., 2013, Merkle et al., 2017).

329 The emergence of variant cells in PSC cultures inevitably entails interactions of variants with their
330 wild-type counterparts, as the two populations share their environment and some of their cell-cell
331 contacts. The nature of these interactions can determine the fate of wild-type cells in a non-cell
332 autonomous manner, thereby impacting on the dynamics of the variant's overtake of cultures (**Figure
333 3**). Some of the commonly occurring PSC variants were shown to suppress the growth of wild-type
334 populations by inducing apoptosis in their neighbouring wild-type cells (Price et al., 2019), in a
335 manner similar to the phenomenon of cell competition described in other model systems (Bowling et
336 al., 2019). In PSC cultures, a differential sensitivity of wild-type and variant PSC to mechanical
337 pressures imposed by cell crowding allowed variants to effectively eliminate wild-type cells from
338 mixed cultures, therefore enhancing the ability of variants to rapidly achieve the clonal dominance
339 (Price et al., 2019). Therefore, consideration of cell interactions, in addition to cell autonomous
340 mechanisms, is needed in developing effective strategies for prevention of growth supremacy of
341 variant cells.

342

343 **Driver genes**

344 The simplest working hypothesis to account for the recurrent selection of a particular chromosomal
345 variant is that it is the altered expression of a single 'driver' gene located in the variant region that
346 provides a growth advantage by altering a cell's behaviour in response to proliferation, differentiation
347 or cell death cues (**Figure 3**). It is, of course, possible that interaction of multiple linked genes in a
348 particular chromosomal rearrangement, or indeed alterations to the chromatin architecture itself,
349 may be responsible. Nevertheless, most studies have focused on seeking a single driver gene.

350 Often the size of the genomic region affected is too large to home in on a likely candidate, but in the
351 case of amplifications affecting chromosome 20, a common minimal amplicon of 0.55Mb was
352 identified in the pericentromeric region of the long arm in all reported examples (Amps et al., 2011).
353 Within this minimal amplicon, containing only thirteen annotated genes, *BCL2L1* was a likely
354 candidate driver gene as its anti-apoptotic splice variant, BCL-XL, is expressed in human PSC (Amps
355 et al., 2011). Experiments in which cells carrying a gain chromosome 20, or that had been
356 transfected with a *BCL2L1*-over expressing vector, were mixed with diploid cells, confirmed that

357 *BCL2L1* and its BCL-XL product was indeed the driver providing a selective growth advantage by
358 blocking apoptosis (Nguyen et al., 2013, Avery et al., 2013).

359 Like chromosome 20, a common minimal amplicon has also been identified on the long arm of
360 chromosome 1 (Baker et al., 2016)(E. McIntire et al, International Society for Stem Cell Research
361 Meeting abstract). A likely candidate driver gene located in this region is *MDM4,* which regulates
362 p53 by suppressing its response to cellular stresses and increasing the threshold to apoptosis (Haupt
363 et al., 2019). Since recurrent dominant negative mutations of *TP53* provide a growth advantage to
364 human PSC (Merkle et al., 2017) it is likely that dysregulation of other genes, such as *MDM4*, that
365 affect apoptosis through p53 would confer a similar growth advantage. On the long arm of
366 chromosome 17, another anti-apoptotic gene, *SURVIVIN (BIRC5)*, encoded in the chromosome
367 17q25.3 region, has been proposed since its inhibition leads to apoptosis of human PSC and cancer
368 cells (Blum et al., 2009, Mesri et al., 2001, Ma et al., 2006, Yang et al., 2004). On the other hand,
369 other candidate genes encoded on chromosome 17 include *WNT3* and *WNT9B*, suggested by their
370 involvement in the altered patterns of differentiation of cells carrying a gain of the long arm of
371 chromosome 17 (Lee et al., 2015).

372 Interest in gains of chromosome 12 has a long history since testicular germ cell tumors almost always
373 have a gain of the short arm, mostly as an isochromosome (Atkin and Baker, 1982), or more rarely
374 as an interstitial amplification (Rodriguez et al., 2003, Korkola et al., 2006). However, there is no
375 definitive evidence to identify the specific driver gene, either for the progression of germ cell tumors,
376 or for the appearance of variant human PSC with a gain of chromosome 12. An obvious candidate
377 driver gene on the short arm of chromosome 12 is *NANOG*, given its central role in maintaining
378 pluripotency, and that its over-expression inhibits differentiation (Chambers et al., 2007). Also, over
379 expression of NANOG does allow human ESC to efficiently form colonies at low density, which is
380 normally associated with extensive apoptosis, perhaps mediated by downregulating LECTIN1, which
381 normally promotes apoptosis, and upregulating HSPA1A, which inhibits apoptosis (Darr et al., 2006).
382 Indeed, *NANOG* is located in a minimal amplicon that has been identified in germ cell tumors,
383 chromosome 12p13.31, but so are two other genes, *DPPA3* and *GDF3*, that also may affect the
384 behaviour of human PSC (Korkola et al., 2006). However, a different minimal amplicon has also
385 been reported in human germ cell tumors, at 12p11.2–p12.1, in which a number of other genes have
386 been highlighted, such as the oncogene, *KRAS* (Rodriguez et al., 2003).

387 Although less frequent, deletions may promote enhanced survival through copy number loss of pro-
388 apoptotic genes. The BCL-2 apoptotic pathway is controlled by interactions between pro- and anti-
389 apoptotic protein family members. Human ESC show elevated expression of the pro-apoptotic
390 genes *NOXA, BIK, BIM, BMF* and *PUMA*, which may contribute to their low apoptotic threshold
391 (Madden et al., 2011, Liu et al., 2013, Dumitru et al., 2012). The two of these most highly expressed
392 in human PSC, *NOXA* and *BIK*, are located in chromosomal regions, 18q21.32 and 22q13.2, that do
393 undergo recurrent deletion. Deletion of *NOXA* by genetic manipulation decreases the sensitivity of

394 human PSC to mitotic errors, thereby increasing the survival of aneuploidy cells (Zhang et al., 2019),
395 and improves survival during cell dissociation, similar to the overexpression of the anti-apoptotic
396 proteins, BCL-2 and BCL-XL (Ohgushi et al., 2010, Ardehali et al., 2011).

397

398 **ACQUISITION OF MUTATIONS**

399 **Mutation Rate in PSC**

400 Whereas the mechanisms by which genetic variants offer cells a selective growth advantage are
401 relatively easy to assess and have been extensively studied, addressing the mechanisms that drive
402 the appearance of the variants in the first instance is more problematic: Mutations occur
403 stochastically and at low frequency in single cells within much larger populations, so that by the time
404 they become detectable the frequency of mutation may have been grossly distorted by the effects of
405 selection. To overcome this problem, we recently adopted a clonogenic strategy in which a single
406 cell was isolated and allowed to expand as a clonal colony for a fixed time, after which the clone was
407 subcloned, with about 20 subclones being isolated and, after expansion, subjected to whole genome
408 sequencing (Thompson et al., 2020). Using this approach, in which most of the mutants that arose
409 were in genes and locations unlikely to result in growth advantage or disadvantage, we estimated
410 the mutation rate of two human, clinical grade ESC lines, MShef4 and MShef11, as 0.37 $x10^{-9}$ and
411 $0.28x10^{-9}$ SNVs per base pair, per day, respectively, equating to approximately 0.30 $x10^{-9}$ and
412 $0.23x10^{-9}$ SNVs per cell division, respectively, given that the cell cycle time of human PSC, in our
413 experience, approximates 20 hours (+/- 2 hours) (Barbaric et al., 2014). This rate was not affected
414 by the use of the Rho associated coiled coil containing protein kinase (ROCK) inhibitor, Y-27632,
415 commonly used in human PSC culture (Watanabe et al., 2007) The frequency of INDELS was 10-
416 fold lower. These low rates are comparable with another study of human iPSC in which the mutation
417 rate was estimated to be 0.18 x 10^{-9} SNV per base-pair, per cell division which was considerably
418 lower than in the endothelial progenitor cells (Rouhani et al., 2016). These mutation rates in human
419 PSC contrast with an estimated rate of 2.66 x 10^{-9} mutations per base pair, per mitosis in somatic
420 cells (Milholland et al., 2017). In another more limited study of a human ESC line, a slightly higher
421 mutation rate of 1 x 10^{-9} SNV per base-pair, per cell division, but again this was much lower than an
422 estimate of a corresponding somatic cell in the same study (Kuijk et al., 2018), while in a study of a
423 single locus, *Aprt*, in mouse ESC, the mutation rate was estimated to be 10 fold lower than in
424 corresponding somatic cells (Cervantes et al., 2002). These low rates are consistent with the
425 infrequency of recurrent point mutations observed in PSC lines: for example Merkle et al (2017)
426 (Merkle et al., 2017) only observed mutations in TP53 in five out of 140 human PSC lines.

427 In our study of the MShef4 and MShef11 human ESC lines (Thompson et al., 2020), the mutation
428 rate was similar across all chromosomes, with no obvious hotspots, with the exception of a slightly
429 raised rate on the X chromosome, which might have been a consequence of both lines being male.
430 Nevertheless, the mutation rate was significantly higher in intergenic regions than in exons and

431 introns, suggesting an influence of chromatin structure on mutation. Further, the predominant
432 mutation signatures that we detected were consistent with oxidative damage being the predominant
433 cause of mutation and, indeed, the mutation rate for both SNV and INDELS was reduced by about
434 50% when the cells were maintained under low (5%) oxygen atmospheres.

435

436 **DNA Replication Stress and Mitotic Errors**

437 While many SNV in PSC, as in other cultured cells (Petljak et al., 2019, Kucab et al., 2019, Viel et
438 al., 2017), are caused by misincorporation of bases due to oxidative stress, the relatively rapid cell
439 cycle of PSC might also expose them to high levels of DNA replication stress, characterised by
440 reduced rates of DNA replication together with stalling and collapse of replication forks (Bartkova et
441 al., 2005, Gorgoulis et al., 2005). Errors in the repair of resulting double stranded DNA breaks could
442 then lead to chromosomal rearrangements (Cannan and Pederson, 2016). Self-renewal of human
443 PSC is characterised by an abbreviated G1 phase that bypasses the RB1-E2F checkpoint due to
444 the high expression of cyclin D2 and its CDK4 partner together with the constitutive expression of
445 cyclin E, which together maintain RB1 in a hyperphosphorylated and inactive state (Becker et al.,
446 2006, Becker et al., 2010, Filipczyk et al., 2007). Using DNA fibre assays we have recently found
447 that, in comparison to isogenic somatic cells, human PSC do exhibit the features of DNA replication
448 stress, including slower DNA replication speeds with evidence of stalled replication forks, and
449 replication initiating from quiescent replication origins (**Figure 4**) (Halliwell et al., 2019). In parallel
450 we also observed more extensive replication-associated DNA damage in the PSC compared to
451 somatic cells, as has also been reported by others (Simara et al., 2017, Vallabhaneni et al., 2018).

452 A similar situation pertains in many cancers where cyclin E is frequently over-expressed and RB1-
453 E2F is constitutively activated (Akli and Keyomarsi, 2003). One of the consequences of this is
454 replication stress, double stranded breaks and genetic instability (Bester et al., 2011, Burrell et al.,
455 2013, Frame et al., 2006, Pickering and Kowalik, 2006). In mouse ESC, also, molecular hallmarks
456 of replication stress are almost identical to those observed when oncogenes, such as cyclin E, are
457 dysregulated in somatic cells (Ahuja et al., 2016), suggesting that atypical cell cycle control with
458 consequent susceptibility to DNA replication stress and genomic damage in PSC parallels the
459 oncogene-induced DNA damage model for cancer development and progression (Halazonetis et al.,
460 2008).

461 Replication stress induced from oncogene expression can lead to nucleotide deficiency and collision
462 of replication forks with transcription complexes (Jones et al., 2013, Bester et al., 2011).
463 Supplementing cancer cells or primary cell lines that overexpress oncogenes, such as cyclin E, with
464 nucleosides has been found to alleviate replication stress and its associated DNA damage and
465 genetic instability in these cases (Bester et al., 2011, Burrell et al., 2013). In a similar manner, we

466 have recently found that exogenous nucleosides increase the rate of replication fork progression and
467 decrease DNA damage in human PSC cultures (**Figure 4**) (Halliwell et al., 2019).

468 While chromosomal non-dysjunction and numerical instabilities may be the product of merotelic
469 kinetochore attachment, in which the microtubules from both poles bind to the same sister chromatid,
470 leading to lagging and potential mis-segregation of chromosomes (Cimini et al., 2001), the
471 persistence of DNA replication defects from S phase into mitosis can also result in the formation of
472 mitotic errors that are a source of chromosomal instabilities (Burrell et al., 2013). Under-replicated
473 regions can interlink sister chromatids during segregation forming anaphase bridges that are prone
474 to breakage forming double stranded breaks (Chan et al., 2009). Often, to prevent anaphase
475 bridges, nucleases cleave the DNA that again generates double stranded breaks (Naim et al., 2013).
476 Further, the condensation of chromosomes that harbour replication intermediates are particularly
477 prone to breakage (Lukas et al., 2011). These double stranded breaks that result from replication
478 intermediates in mitosis are the substrates for genetic instability caused by error induced repair.

479 By fluorescently labelling human PSC with histone H2B-mCherry it was observed that 30% of
480 mitoses were abnormal including a high proportion with lagging chromosomes and anaphase bridges
481 (Zhang et al., 2019), a level substantially higher than that observed in somatic cell lines (Lamm et
482 al., 2016). In comparison to somatic cell lines, diploid human PSC show condensation defects that
483 result in partially condensed and entangled chromosomes (Lamm et al., 2016). Supplementing
484 cultures with exogenous nucleosides alleviated replication stress and decreased the frequency of
485 mitotic errors, providing further evidence that these are linked in human PSC (Halliwell et al., 2019),
486 as well as providing an approach to reducing their appearance in PSC cultures. However, it should
487 be noted that the continued occurrence of mitotic errors, even with the addition of nucleosides
488 suggests that there are other factors driving their occurrence.

489

490 **Response to genomic damage**

491 Human PSC deploy a number of mechanisms to minimise the effective mutation rate that otherwise
492 might be anticipated from their high susceptibility to DNA damage. Genes involved in various repair
493 pathways show increased expression compared to somatic cells (Maynard et al., 2008, Momcilović
494 et al., 2009), and nucleotide excision repair, base excision repair, and the resolution of inter-strand
495 crosslinks caused by ionising radiation all have all been reported to be faster in human PSC than
496 somatic cell lines (Luo et al., 2012, Hyka-Nouspikel et al., 2012, Maynard et al., 2008). PSC also
497 tend to repair double strand break using homologous recombination, which is prone to less errors
498 than non-homologous end joining (Adams et al., 2010a), although they do also utilise a higher fidelity
499 system of non-homologous end joining that is independent of DNA-PKc and ATM (Adams et al.,
500 2010b). Further, in response to the formation of reactive oxygen species as a by-product of
501 respiration, and consequent oxidative stress, PSC express higher levels of SOD2 and GPX2 anti-
502 oxidant enzymes compared to differentiated lines (Saretzki et al., 2008).

503 Nevertheless, human PSC generally activate apoptosis when exposed to lower doses of genotoxic
504 insults than do somatic cells, suggesting that a low apoptotic threshold is the key element in their
505 response to genomic damage. After human PSC are exposed to ultraviolet C radiation to induce
506 nucleotide base adducts or DNA breaks they respond with extensive apoptosis even at mild doses
507 that have little effect on somatic cell lines (Hyka-Nouspikel et al., 2012, Luo et al., 2012, Simara et
508 al., 2017). Similarly, the treatment of human PSC with cis-platinum or thymidine to initiate replication
509 block (Desmarais et al., 2012, Desmarais et al., 2016), or with nocadazole to induce mitotic block
510 (Zhang et al., 2019) also elicits an extensive apoptotic response in contrast to the response by
511 somatic cells, while PSC also efficiently activate apoptosis in response to oxidative stress (Saretzki
512 et al., 2008). The particular sensitivity of embryonal carcinoma cells, the malignant PSC of
513 teratocarcinomas, to drugs such as cis-platinum (Oosterhuis et al., 1984, Einhorn and Donohue,
514 1977), a DNA cross linking agent, makes germ cell tumors one of the most treatable forms of solid
515 cancer, most likely reflecting this particular low apoptotic threshold.

516 An atypical cell cycle checkpoint control mechanism most likely underlies the low apoptotic threshold
517 of human PSC. In response to DNA damage, human PSC fail to activate p21, which is normally
518 required to execute the G1/S checkpoint, providing less time for repair before apoptosis is initiated
519 in a p53 dependant manner (Hyka-Nouspikel et al., 2012, Hong and Stambrook, 2004, Momcilović
520 et al., 2009). Further, in response to DNA replication stress caused by high levels of thymidine, or
521 the presence of cis-platinum, human PSC, unlike somatic cells, do not activate ATR-CHK1, while
522 foci of RPA, which binds to single stranded DNA at stalled replication forks, are not formed: instead
523 the cells commit to apoptosis (Desmarais et al., 2012, Desmarais et al., 2016). Human PSC also
524 undergo extensive apoptosis in response to mitotic stress which may safeguard the genome from
525 abnormal mitosis by clearing the effected cell from the cell pool (Zhang et al., 2019). Collectively,
526 these studies support a model in which genomic stability, and the particularly low observed mutation
527 rate of PSC is primarily maintained by a low apoptotic threshold. Consequently, blocking apoptosis
528 seems to be the most likely mechanism that provides selective growth advantage for the common
529 genetic variants found in human PSC: the two driver genes so far identified, *TP53* and *BCL2L1*, both
530 act to inhibit apoptosis, while other proposed candidates, MDM4 and SURVIVIN, are also anti-
531 apoptotic.

532 This low apoptotic threshold of human PSC may reflect their relationship to the early embryo in which
533 the need for rapid cell doublings is accomplished by the lack of cell cycle checkpoints, rendering the
534 cells particularly susceptible to errors in DNA synthesis and mitosis, which could be catastrophic for
535 subsequent embryonic development. Indeed, almost half of human embryos fail to survive due to
536 chromosomal instability, which does not seem to be a mere artefact of *in vitro* fertilisation (van
537 Echten-Arends et al., 2011, Munné et al., 2019, Starostik et al., 2020). It has been observed that
538 the mosaic embryos that survive to the blastocyst stage undergo “genetic normalization” when
539 cultured under routine IVF conditions (Brezina et al., 2011). The mechanism of genetic normalization

540 is still widely debated although, in the mouse, activation of apoptosis during the later pre-implantation
541 stages may allow for the removal of aneuploid cells from the developing embryo (Kops et al., 2004,
542 Bolton et al., 2016). This model is supported by observations that the proportion of aneuploidy in
543 the inner cell mass is reduced, whereas in the trophectoderm it is enriched as development proceeds
544 (Hardy, 1997). However, this is still widely debated as apoptosis is a feature of all embryos, and
545 may be a mechanism for maintaining cellular homeostasis regardless of their genomic state (Haouzi
546 and Hamamah, 2009).

547 Another context in which the apoptotic response of human PSC to DSB induction is of central
548 importance, is the process of genome editing. The rising prominence of genome editing
549 technologies, in particular CRISPR/Cas9-based methods, has fuelled efforts aimed at, for example,
550 correcting germline mutations in PSC to allow autologous cell therapy, or removing HLA antigens to
551 reduce the need for immunosuppressants in transplanted patients. Crucially, as gene editing relies
552 on the induction of DSB by nucleases, the edited PSC undergo high levels of apoptosis in
553 comparison to their unedited counterparts (Ihry et al., 2018). The rate of cell death was shown to be
554 similar between different, edited PSC, regardless of whether the targeted gene was expressed in
555 PSC or whether it was non-expressed and dispensable for PSC maintenance. This observation
556 supports the view that the induction of DSB during the gene editing process commits PSC to
557 apoptosis (Ihry et al., 2018). Mechanistically, DSB induction by Cas9 was shown to trigger
558 differential gene expression in edited cells, most notably by promoting the TP53 transcriptional
559 response (Ihry et al., 2018). In line with the importance of TP53 activation during the genome editing
560 process, performing the PSC genome editing in cells with genetically inactivated *TP53* reduced the
561 levels of cell death and improved the efficiency of PSC genome editing (Ihry et al., 2018). While
562 genetic inactivation of TP53 is deemed too risky for editing of cells destined for clinical use, transient
563 TP53 inactivation has been proposed as a possible alternative (Schiroli et al., 2019). Further work
564 will need to carefully address this possibility, to ensure that the transient TP53 inactivation does not
565 inadvertently select for *TP53* genetic mutants. Relevant to this notion is the recent data
566 demonstrating the emergence of *TP53*-inactivating mutations in cancer cell lines during the
567 CRISPR/Cas9 genome editing process (Enache et al., 2020).

568

569 **CONCLUSIONS**

570 Efforts to collect and catalogue genetic variation in PSC over the last two decades demonstrated
571 that particular variants do arise in cultures and are sometimes difficult to detect because of the
572 limitation of sensitivity of detection methods. Nonetheless, it is reassuring that the rate of mutation
573 in PSC cultures is low compared with somatic cells. Indeed, based on the data from large-scale
574 retrospective studies, such as the ISCI (Amps et al., 2011), and on the direct measurements of the
575 mutation rates in PSC (Thompson et al., 2020), there is no evidence to suggest that PSC genomes
576 are particularly unstable. Rather, the clonal expansion of genetic variants against the backdrop of

577 low mutation rates, can be explained by the effect of selective pressures operating in PSC cultures.
578 Optimizing culture conditions and protocols to minimize the growth advantages of the common
579 variants is, therefore, a key route to maintaining the genetic integrity of PSC lines. However, the
580 predominant selective force dominating PSC cultures appears to be a high rate of apoptosis (Dumitru
581 et al., 2012, Barbaric et al., 2014). Apoptosis seems to be a default fate choice of PSC in many
582 different scenarios, including a response of cells to genome damage or mitotic stress (Desmarais et
583 al., 2012, Desmarais et al., 2016, Dumitru et al., 2012, Zhang et al., 2019), most likely reflecting its
584 function of maintaining the genetic integrity of the early embryo. Consequently, optimising culture
585 conditions should entail removing the apoptotic stimuli, but not blocking apoptosis *per se* which
586 would be counterproductive.

587 Armed with a knowledge of recurrent karyotypic and sequence changes, our attention now needs to
588 turn to finding ways of minimizing their occurrence by lowering the genome damage and reducing
589 the selective pressures. In that respect, the observations that mutation rates can be decreased by
590 growing cells under low oxygen (Thompson et al., 2020) and that replication-stress induced genome
591 damage can be alleviated by addition of exogenous nucleosides (Halliwell et al., 2019) provide
592 foundations for optimised culture conditions of PSC. Further work should also address the
593 contribution of epigenetic variants to aberrant PSC phenotypes, as the understanding of epigenetic
594 variation in PSC cultures remains limited.

595 Finally, the field awaits deciphering of the functional consequences of the karyotype and sequence
596 changes on PSC traits and on the behaviour of their differentiated derivatives. Interpreting the role
597 of specific variants is complicated by the fact that their consequences are likely to be context-
598 dependent. For example, a mutation in a gene expressed specifically in an endodermal lineage may
599 have little impact on the clinical application of neuronal cells. To aid these analyses, ISCI is
600 proposing an international study group to collate and monitor evidence of genetic variants in PSC
601 and their potential consequences (Andrews et al., 2017). However, it should be noted that the
602 success of this approach requires a concerted effort within the field to perform routine monitoring
603 and report the presence of genetic variants, thereby allowing retrospective analyses of their effects.
604 We envisage that these initiatives, in a synergy with cancer genome efforts, would provide a rational
605 strategy to assess the potential risk of different mutations, a necessary requirement for routine, safe
606 clinical implementation of cellular therapies.

607

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1056 **FIGURE LEGENDS**
1057
1058 **Figure 1: Appearance of mutations in human PSC involves both mutation and**
1059 **selection.**
1060 Human PSC are subject to the full range of mutations seen in other systems, including single
1061 base changes and small insertions and deletions (INDELS) (Thompson et al., 2020), as well
1062 as larger scale genomic rearrangements with gains or losses of whole chromosomes or
1063 chromosomal fragments that alter the number of copies of whole sets of genes, and
1064 consequently their levels of expression (Enver et al., 2005). The most commonly seen
1065 chromosomal rearrangements are illustrated. Generally, in the absence of a clonogenic
1066 bottleneck, these mutations will never be detected unless they offer the mutant cell a
1067 selective growth advantage (Olariu et al., 2010).
1068
1069 **Figure 2: Nature of tumors derived from pluripotent stem cells**
1070 PSC produce tumours that may contain both undifferentiated PSC together with their
1071 differentiated derivatives, in which case the tumor is termed a 'Teratocarcinoma' and is
1072 regarded as highly malignant. If the PSC fully differentiate, so that the tumor contains only
1073 differentiated derivatives, it is termed a 'Teratoma' and is generally regarded as benign and
1074 not malignant (Damjanov and Andrews, 2007a, Damjanov and Andrews, 2016). However,
1075 a caveat is that some of the differentiated derivatives may develop into a secondary
1076 malignancy corresponding to their particular cell type, and this may be driven by a mutation
1077 present in the parent PSC. In addition to somatic derivatives, PSC may also generate
1078 primitive endoderm elements which are precursors of highly malignant yolk sac carcinomas
1079
1080 **Figure 3: Mechanisms of Variant Growth Advantage**
1081 A variety of mechanisms can be envisaged by which variant cells could gain a growth
1082 advantage over wild type cells. **Cell autonomous mechanisms:** mutation **(a)** drives faster
1083 cell cycle, or **(b)** blocks differentiation, or **(c)** blocks apoptosis. **Cell interactive**
1084 **mechanisms**: mutation **(d)** causes variant cell to inhibit the growth of its wild-type
1085 counterpart (Price et al., 2019) or **(e)** alters the patterns of differentiation. The latter could
1086 generate a selective advantage either if certain differentiated derivatives produced factors
1087 that promote differentiation (advantage would derive from blocking such lineages) or
1088 produced factors that blocked general differentiation (advantage would derive from
1089 enhancing differentiation to such lineages).
1090
1091 **Figure 4: Overview of the origins of mutation in human PSC. a)** Somatic cellular
1092 proliferation occurs predominantly without replication stress, allowing for faithful cell division.
1093 However, should replication stress occur, somatic cells can respond with cellular
1094 senescence, apoptosis or DNA repair. **b)** Human pluripotent stem cells proliferate rapidly
1095 and are susceptible to replication stress, DNA damage and mitotic errors. However, they
1096 display a low mutation rate, which is reconciled by responding to genetic stresses with
1097 apoptosis and efficient DNA repair. **c)** Culture of human PSC with exogenous nucleoside
1098 alleviates replication stress, DNA damage and mitotic errors. Alleviating genetic stress
1099 minimises apoptosis, enhancing growth rate and removing the selective advantage of anti-
1100 apoptotic mutations.
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1102
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1104

BOX 1. Detection of Genetically Variant Cells

Routine monitoring of PSC cultures for the appearance of genetic variants is essential: the proportion of some common variants in a culture can rise from undetectable to 100% within 5 -10 passages (Olariu et al., 2010). Different screening methods have different advantages and limitations, including the minimum proportions of variant cells that can be detected in mosaic populations, and whether prior knowledge of the variant to be detected is required. Other factors to consider are the types of variant detectable, cost, speed and whether specialist facilities and expertise are required. At a minimum we would recommend regular screening of cultures for common variants using the PCR method, which is rapid and can easily be carried out in most laboratories, backed up at key decision points in specific experiments with more in-depth analysis using other methods as appropriate.

- **Karyotyping by G banding:** Indiscriminate view of structural and numerical chromosomal abnormalities greater than 5Mb (Steinemann et al., 2013). Limited to analysis of proliferating cells, but the only convenient method to detect balanced genomic rearrangements. Sensitivity and precision can be enhanced by use of fluorescent hybridization probes. Minimum mosaicism detected: 18% (routine analysis of 30 metaphase spreads), 6% (analysis of 100 metaphase spreads) (Baker et al., 2016).
- **Array Comparative Genome Hybridisation (aCGH) and SNP array:** Global screening detects copy number variants down to 1Kb. Minimum mosaicism detected: 10-15% for small copy number variants. (Valli et al., 2011).
- **Interphase Fluorescent In Situ Hybridisation (FISH):** Hybridisation of fluorescent probes to highly complementary nucleic acid sequences in interphase cells. Requires prior knowledge of the copy number variant to be detected. The presence of false negatives dependent upon the type of variant restricts the sensitivity of detection: Minimum mosaicism detected depends upon the number of nuclei assessed and the size of the CNV: ~5% is possible for gains of whole chromosome arms, with 100 nuclei assessed, but this falls markedly for smaller CNV (Baker et al., 2016).
- **qPCR based assays:** Rapid method suitable for any well-equipped molecular cell biology laboratories. Requires prior knowledge of the CNV to be detected. Minimum mosaicism detected: ~5% (Baker et al., 2016).
- **Expression karyotyping:** Regions of CNV are detected based on their global gene expression from RNA-seq data in comparison to a diploid calibrator sample. Requires no prior knowledge of the CNV to be detected, although it is only capable of detecting chromosomal aberrations greater than ~10Mb with a sensitivity of ~30% (Mayshar et al., 2010).

1140 • **eSNP Karyotyping:** An adaptation of expression karyotyping with allele bias. This approach
1141 does not require a diploid calibrator sample for CNV detection. Resolution of eSNP
1142 karyotyping depends on the genomic region and sequencing depth, yet it comfortably
1143 detected amplifications to entire chromosomes or chromosome arms. The eSNP
1144 karyotyping approach shows comparable sensitivity to other DNA based array approaches
1145 (~30%) (Weissbein et al., 2016).

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1168 **BOX 2: Features of common genetic changes in PSC.**
1169
1170 **Gains:**
1171
1172 **Chromosome 1q**
1173 • Typically acquired as a structural chromosome rearrangement (unbalanced translocation or
1174 interstitial duplication) (Amps et al., 2011, Baker et al., 2016)
1175 • Minimal region: 1q25-q41 (Baker et al., 2016)
1176 • Potential driver gene: *MDM4*
1177 • Association with cancer: Wilms' tumor (Chagtai et al., 2016), multiple myeloma (Walker et
1178 al., 2010) and intracranial ependymomas (Kilday et al., 2012)
1179
1180 **Chromosome 12p**
1181 • Typically acquired as a whole chromosome trisomy, although a number of studies have also
1182 reported a partial trisomy of the short arm of chromosome 12 (Amps et al., 2011, Baker et
1183 al., 2016)
1184 • Minimal region: 12p11-pter (Baker et al., 2016)
1185 • Potential driver gene: *NANOG*
1186 • Functional consequences observed to date: increased proliferation rate due to increased
1187 replication, reduced propensity for spontaneous differentiation and apoptosis (Ben-David et
1188 al., 2014)
1189 • Association with cancer: testicular germ cell tumors (Atkin and Baker, 1982)
1190
1191 **Chromosome 17q**
1192 • Typically acquired as a structural chromosome rearrangement (unbalanced translocation or
1193 interstitial duplication) (Amps et al., 2011, Baker et al., 2016)
1194 • Minimal region: 17q25-qter (Baker et al., 2016)
1195 • Potential driver gene: *SURVIVIN* (*BIRC5)*
1196 • Functional consequences observed to date: growth advantage in undifferentiated cultures
1197 (Olariu et al., 2010) due to enhanced proliferation (Lee et al., 2015); faster differentiation to
1198 mesodiencephalic dopaminergic neural cells compared with wild-type cells (Lee et al.,
1199 2015)
1200 • Association with cancer: testicular germ cell tumors (Kraggerud et al., 2002) and
1201 neuroblastoma (Bown et al., 2001)
1202
1203 **Chromosome 20q**
1204 • Typically acquired as an interstitial duplication (Amps et al., 2011)
1205 • Minimal region: 20q11.21 (Amps et al., 2011)
1206 • Driver gene: *BCL2L1* (Avery et al., 2013, Nguyen et al., 2013)
1207 • Functional consequences observed to date: growth advantage due to reduced propensity
1208 for apoptosis (Avery et al., 2013, Nguyen et al., 2013), reduced dependence on bFGF
1209 (Werbowetski-Ogilvie et al., 2009), delayed differentiation to neural lineages (Werbowetski-
1210 Ogilvie et al., 2009) and reduced efficiency of neuroectodermal lineage commitment
1211 (Markouli et al., 2019)
1212 • Association with cancer: colorectal cancer (Nguyen and Duong, 2018)
1213
1214
1215 **Losses:**
1216
1217 **Chromosome 10p**
1218 • Typically acquired as an interstitial deletion (Amps et al., 2011)
1219 • Minimal region: 10p13-pter (Amps et al., 2011, Baker et al., 2016)
1220 • Association with cancer: melanoma (Robertson et al., 1999) and glioblastoma (Kimmelman
1221 et al., 1996)
1222

1223

1224 **Chromosome 18q**

1225 - Typically acquired as an interstitial deletion (Amps et al., 2011)
1226 - Minimal region: 18q21-qter (Amps et al., 2011, Baker et al., 2016)
1227 - Association with cancer: colorectal carcinoma (Popat and Houlston, 2005)

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